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Chemically modified human hemoglobin is currently being studied as a potential blood substitute for use in military and emergency medical applications. We have tested one form of modified hemoglobin, DBBF-Hb, against normal Hb in order to determine its ability to promote hemoglobin-driven bacterial infections. Using an experimental model of E. coli peritonitis we have determined that DBBF-Hb is equally likely as unmodified Hb (on a mole to mole basis) to lead to a fatal outcome in this model. Further investigations were undertaken to elucidate the molecular mechanism of these hemoglobin-drinve bacterial infections. The strains of E. coli that exhibit the hemoglobin-adjuvant effect are resistant to phagocytosis by peritoneal macrophages. This feature may explain why hemoglobin is necessary (nutritional iron) but not sufficient for the promotion of E. coli infections. We are currently examining the composition of the outer membrane proteins in the hemoglobin-adjuvant strains. In addition, using a molecular biological approach, we are attempting to clone the gene(s) responsible for the hemoglobin-adjuvant phenotype in E. coli. Further understanding of the mechanism of hemoglobin driven bacterial infections may enable the rational design of a safe blood substitute

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Abbreviations:

Escherichia coli - *E. coli*
deferoxamine - DFO
phosphate buffered saline - PBS
high molecular weight deferoxamine - HMW-DFO
polyacrylamide gel electrophoresis - PAGE
stroma free-hemoglobin - SF-Hb
bis(3,5-dibromosalicyl) fumarate hemoglobin - DBBF-Hb
gallium - Ga
nitric oxide - NO•
nitric oxide synthase - NOS
lethal dose 50% - LD_{50}
intraperitoneal - IP
brain heart infusion - BHI
Swiss Webster - SW
minimal medium - M9
heat inactivated - HI
colony forming unit - cfu
ampicillin resistance - Amp^r
polymorphonuclear leukocyte - PMN

INTRODUCTION

These investigations were designed to test the possibility that modified hemoglobins - under development as potential human blood substitutes - might increase the pathogenicity of certain strains of *Escherichia coli* (perhaps through providing nutrient iron which is ordinarily present in only trace amounts *in vivo*). If this is true, the implementation of strategies for the sequestration of hemoglobin-derived iron, might prevent any 'adjuvant' effect of modified hemoglobins on bacterial pathogenicity. Overall goals of the research (as presented in the initial grant application) included:

1. Determination of factors which affect the iron-dependent growth of bacteria in simple *in vitro* systems.

2. Investigation of a chemically modified hemoglobin, Bis (3,5-dibromosalicyl) fumarate-hemoglobin [DBBF-Hb] being developed for use as blood substitutes as potential sources of nutrient iron for bacterial growth and as adjuvants in an experimental peritonitis model.

3. Determination of the effects of chemical modification of hemoglobin on the interaction of DBBF-hemoglobin and human haptoglobin.

4. Testing of new strategies for the pharmacologic sequestration of iron and, consequently, limitation of bacterial pathogenicity. Implicit in this last goal is a search for the mechanisms whereby hemoglobin enhances bacterial lethality, because these mechanisms must be understood before we can design rational modes of therapeutic intervention.

BODY

To accomplish these aims we:

- acquired several strains of *E. coli* which do or do not exhibit a "hemoglobin adjuvant effect" (i.e., become much more lethal in the presence of free hemoglobin or other iron sources),
- measured the production of lytic toxins (so-called "hemolysins") by these strains when grown in the presence and absence of control and cross-linked hemoglobins,
- measured the effects of hemoglobin preparations on the *in vitro* growth of these strains in iron-poor media,
- acquired the first test DBBF-hemoglobin preparations from LAIR,
- successfully developed a new *E. coli* peritonitis model employing mice rather than rats,
- tested the adjuvanticity of DBBF-hemoglobin preparation versus stroma free hemoglobin in rodent peritonitis models,
- began to dissect the pathophysiologic bases for the hemoglobin adjuvant effect,
- showed that stroma free-hemoglobin does not appear to affect the resistance of the adjuvant strains to killing by purified neutrophils or by phagocytic cells (predominantly neutrophils) in while blood,
- showed that stroma free-hemoglobin does *not* mediate the production of a factor by adjuvant stains responsible for the enhanced lethality of these infections,
- stroma free-hemoglobin in the presence of bacteria exhibiting the adjuvant effect, do not prevent the recruitment of phagocytes to the site of bacterial infection,
- immunization against an adjuvant strain reduces mortality in the infection model and cross protects against infection with another adjuvant strain,
- cloned the adjuvant genome into pUC19 and transformed a non-adjuvant strain. We will select for hemoglobin-dependent virulent strains using the infection model.

In addition to the DBBF-Hb supplied to us by LAIR for these studies I wrote to Dr. Stetler, Somatogen, Inc., Boulder, CO. and requested a sample of recombinant hemoglobin (rHb1.1) which has been reported to have markedly reduced renal toxicity and better oxygen affinity characteristics for a hemoglobin blood substitute (A human recombinant haemoglobin designed for use as a blood substitute. Nature 365:258-260, 1992). Unfortunately, Somatogen was unwilling to provide any of their recombinant hemoglobin. Therefore, we were not able to test whether or not this genetically engineered hemoglobin behaves differently from normal or chemically cross-linked hemoglobin (DBBF-Hb) in our experimental infection model.

Summary of Results:

1. Cytochrome c, a protein with covalently bound heme, as an adjuvant.

In an attempt to address the nature of the role of hemoglobin iron in the bacterial adjuvant effect we tested another protein of approximately the same molecular mass as a hemoglobin monomer which also contains a heme prosthetic group, namely, cytochrome c. The importance difference between these two proteins is that the heme group is covalently attached to the protein moiety in the case of cytochrome c and should not be able to dissociate as readily as the heme bound (non-covalently) to hemoglobin. To our surprise were demonstrated that on a mole per mole basis (representing a 1:1 stoichiometry of iron) cytochrome c is as potent an adjuvant as hemoglobin.

2. Does the inhibition of macrophage nitric oxide synthase have an adjuvant-like effect ?

Bacteria introduced into the peritoneal cavity, as in our experimental model encounter resident peritoneal macrophages as the first line of host defense. Peritoneal macrophages are thought to be responsible for phagocytosis and clearance of bacteria in the peritoneal cavity. Macrophages, unlike neutrophils do not contain myeloperoxidase and are therefore not capable of producing hypochlorous acid, a potent bactericidal oxidant. However, macrophages make nitric oxide (NO•) which has been shown to have similarly potent microbicidal activity. Since NO• has been shown to react with hemoglobin iron resulting in the formation of nitrite and nitrate we tested the hypothesis that impairment of the production of NO• by peritoneal macrophages could mimic the adjuvant effect that is seen with hemoglobin. Therefore, before inoculation with adjuvant bacteria a group of mice was pretreated with an inhibitor of the enzyme responsible for the synthesis of NO•. Mice treated in this fashion did not demonstrate any increased mortality following inoculation with adjuvant bacteria. Due to the lack of a positive result we did not further investigate this model.

3. Competitive inhibition of iron-driven infection by gallium.

It is widely held that iron is the key ingredient in hemoglobin that is responsible for the hemoglobin adjuvant effect. One hypothesis of this mechanism is that the hemoglobin iron (as well as free iron) may serve a growth promoting effect in adjuvant strains of bacteria. Since *E. coli* in a low iron environment induce a series of genes responsible for the synthesis of high affinity iron chelators and their receptors we attempted to use gallium as a competitive substrate for this iron acquisition system, thereby preventing the bacteria from assimilating sufficient iron to sustain growth leading to a fatal infection. Gallium, administrated in relatively high doses, does not diminish the level of mortality seen in the hemoglobin or iron driven infections by adjuvant strains of *E. coli*. Thus, we were not able to competitively inhibit either the hemoglobin or iron adjuvant effect with gallium.

4. Do high affinity iron chelators attenuate the adjuvant effect of iron or hemoglobin ?

We have previously demonstrated that haptoglobin was totally effective in preventing the adjuvant effect of hemoglobin with hemoglobin-responsive strains of *E. coli*. This may be due to the increased stability of the heme moiety in the hemoglobin-haptoglobin complex. Thus, the heme or heme-iron may need to dissociate from hemoglobin in order to exert its adjuvant effect. In an attempt to intercept this dissociating iron were used a variety of high affinity iron chelators to bind any free iron and at least in some cases presumably make it unavailable for bacterial utilization. Using deferoxamine and high molecular weight deferoxamine (HMW-DFO) conjugates which were coincidentally administered with hemoglobin or iron and adjuvant bacteria we were unable to demonstrate any effect of these chelators in preventing the iron-driven infections.

5. *In vitro* growth of *E. coli* in deferrated medium: the effect of iron chelates.

In vitro growth studies in deferrated medium in the presence of added iron with and without a variety of high affinity chelators demonstrated that the chelators were able to delay the growth requiring iron for a period of time but even in the case of the high molecular weight deferoxamine (HMW-DFO) iron chelators the bacteria (either by competition with their own siderophores or the degradation of the HMW DFO) were eventually able to obtain sufficient iron for growth.

6. Identification of adjuvant specific outer membrane proteins.

An important aspect of the hemoglobin adjuvant effect is that not all strains of pathogenic *E. coli* are more virulent in the presence of hemoglobin. In our evaluation of 22 clinical isolates of *E. coli* we found only three strains that exhibit a decrease in their LD_{50} of greater than 10 fold in the presence of hemoglobin. With this in mind we decided to examine the production of proteins of the adjuvant strains of *E. coli* when grown in a low iron medium (heat inactivated serum) in the presence and absence of hemoglobin. Our initial experiments were a comparison of protein from extracts of 0065 grown in an iron rich medium (brain heart infusion) and heat-inactivated serum with and without hemoglobin. As demonstrated in the polyacrylamide gel there is a dramatic induction of proteins when 0065 is grown in serum. These proteins correspond to the membrane proteins associated with the siderophore iron acquisition system. However, there was no demonstrable difference between protein profile of the bacteria grown in serum $\pm$ hemoglobin. Since extracts contain approximately 2,000 different protein we used 2 dimensional electrophoresis in order to increase the resolving power of the comparison. Bacteria grown in serum in the presence and absence of hemoglobin did not reveal any major differences in their 2D electrophoresis profile. However, focusing on the outer membrane we have identified two or three proteins that are constituents of the outer membrane of the adjuvant but not non-adjuvant strains of *E. coli*. These proteins may be involved in the antiphagocytic properties of these adjuvant strains. We have raised antibodies to these proteins and have demonstrated that we can block the adjuvant effect by passively immunizing animals with antibodies to the adjuvant bacteria either by directly administering the antiserum or pretreating the bacteria with the antiserum before injection. It should be noted that antiserum against adjuvant strains of *E. coli* cross protect in this model. This suggests that the

adjuvant strains, in spite of having different O, K and M serotypes, may possess a common surface determinant(s) that is important in conferring the adjuvant phenotype.

7. Preincubation of 0065* in heat inactivated serum ± hemoglobin before IP injection into mice.

Preincubation of two adjuvant strains, 0000 and 0065, was carried out in heat-inactivated human serum ± 20 mg/ml of stroma free hemoglobin. The bacteria were washed 3 X in sPBS and 10^6 bacteria were inoculated IP into mice. There was no mortality in any group suggesting that prior exposure to hemoglobin was not sufficient to express the adjuvant phenotype in these two strains.

8. Time course of the growth of *E. coli* (adjuvant and non-adjuvant strains) *in vivo* in the presence and absence of hemoglobin.

In order to better understand the natural history of the bacterial infections of the adjuvant and non-adjuvant strains we decided to look at a subgroup of these strains and compare the number of recoverable viable bacteria from the peritoneal cavity, liver and spleen over a 12 hour time course. We were able to almost quantitatively recover bacteria from the peritoneal cavity at 0 time. Sampling the peritoneal cavity by lavage and homogenizing tissue from liver and spleen demonstrated a dramatic difference between the adjuvant and non-adjuvant strains of *E. coli* during this time course. Briefly, the non-adjuvant strains were largely killed and/or cleared at the 4 hour time point independent of the presence or absence of hemoglobin. On the other hand, the number of recoverable adjuvant bacteria from the peritoneal cavity was the same or increased from the 0 time inoculum. Additionally, the number of adjuvant bacteria at 4 hours appeared to be roughly the same irrespective of the presence or absence of hemoglobin. Yet, after 4 hours there is a dramatic increase in the number of adjuvant bacteria in the presence of hemoglobin.

By 10 or 12 hours the number of adjuvant bacteria in the presence of hemoglobin is greater than the LD_{50} for these bacteria without hemoglobin. This result suggests that it is the overwhelming growth of adjuvant bacteria that eventually causes the demise of the animal and not the production of a potent toxin in the presence of hemoglobin. In sharp contrast, there were virtually no recoverable bacteria in the peritoneal lavage of animals inoculated with non-adjuvant bacteria ± hemoglobin or with adjuvant bacteria in the absence of hemoglobin. The rapid clearance of non-adjuvant bacteria from the peritoneal cavity is consistent with the findings of other investigators regarding the clearance kinetics of bacteria from the peritoneal cavity (Dr. Carol Wells, University of Minnesota, personal communication). Therefore, it appears that the adjuvant strains of *E. coli* are able to colonize the peritoneal cavity and in the presence of hemoglobin grow to a level which is lethal for the animal.

9. Detection of plasmids in *E. coli* strains - 0000, 0002, 0004, 0006, 0064 and 0065 by agarose gel electrophoresis.

All six strains used in these studies were checked for the presence of plasmids by chloramphenicol amplification followed by agarose mini-gel electrophoresis and ethidium bromide staining. Plasmids were detected only the nonadjuvant strains

which suggests that the iron virulence characteristic is not carried by a plasmid in the adjuvant strains.

10. Coinfection model with an adjuvant (0065) and a non-adjuvant strain (0064pUC118).

We designed a coinfection model of an adjuvant and non-adjuvant strain to determine if the adjuvant strain in the presence of hemoglobin caused the impairment of host defense. A non-adjuvant strain (0064) was transformed with pUC-118 amp^r to make possible the determination of the concentration of both strains of bacteria using selective plating. The coinfection of animals with two strains of bacteria revealed that the non-adjuvant strain is rapidly cleared even in the presence of the adjuvant strain and hemoglobin. This suggests that neither the presence of hemoglobin nor the adjuvant strain has a general negative effect on the host defense. In fact, the kinetic of the adjuvant strain + hemoglobin was not affected by the coinfection with the plasmid-containing non-adjuvant strain.

11. *In vivo* bacterial growth studies ± hemoglobin.

In light of the difficulty in performing *in vitro* phagocytosis and killing assays with mouse peritoneal macrophages we decided on a more qualitative approach to the question of whether there is any significant difference between the adjuvant and non-adjuvant strains with respect to phagocytosis by peritoneal macrophages. We injected 10^6 bacteria with and without hemoglobin and after a period of time sacrificed the animal and lavaged the peritoneal cavity. The peritoneal lavage was both plated and a cytospin was prepared. This allowed the comparison of the number of viable bacteria with the location of the bacteria, i.e., are they largely intracellular or extracellular ? We found that the non-adjuvant bacteria were rapidly cleared from the peritoneal cavity as evidenced by both the recovery of only a small number of bacteria and few bacteria observed by microscopy. Interestingly, the non-adjuvant bacteria present in the slides were almost exclusively found inside macrophages. This is in sharp contrast to what was observed with the adjuvant strains of *E. coli*. At short time periods (30 min)there was about 50% ingestion by macrophages. This corresponds with a reduction in the viability of adjuvant bacteria recovered.

12. Autologous and cross immunization with non-adjuvant and adjuvant strains.

The results from the *in vivo* growth and clearance studies revealed that the adjuvant strains persisted in the peritoneal cavity of the mice following injection. The early rate of disappearance was independent on the presence of hemoglobin and always much faster in the nonadjuvant strains. This suggested that the increase in virulence might be due to an inability of the host phagocytes to recognize and phagocytose the adjuvant strains of *E. coli*. Antiphagocytic properties of bacteria are usually associated with the cell wall or outer membrane of the bacteria. Therefore, we hypothesized that the adjuvant strains of bacteria may have an altered outer membrane that confers resistance to phagocytosis. We also hypothesized that this trait may be found on both adjuvant strains, 0000 and 0065. Note: neither of these strains possess K serotypes that would be consistent with a resistance to phagocytosis.

We designed a series of experiments to examine the effect of prior immunization with either nonadjuvant or adjuvant strains upon the subsequent

challenge with adjuvant bacteria in the presence of hemoglobin. We found that immunization with a nonadjuvant strain did not affect the mortality following a challenge with either adjuvant strain (0000 or 0065) in the presence of hemoglobin. However, immunization with an adjuvant strain afforded protection against an autologous challenge as well as challenge with the other adjuvant strain. These results highly suggest that 0000 and 0065 share a common surface determinant that may be involved in their resistance to phagocytosis. Exposure of the adjuvant bacteria to the antibodies against this determinant(s) greatly diminished the virulence of these adjuvant strains.

13. Molecular cloning and selection of the virulence gene in hemoglobin-adjuvant strains of *E. coli*.

Molecular cloning of the virulence gene(s) from the adjuvant strain and insertion into a nonadjuvant strain followed by *in vivo* selection for the adjuvant trait. The following experimental approach for the identification of the virulence gene is based on the assumption that this trait is encoded by a single gene. If this is not the case it is unlikely that this selection technique would be successful. The molecular cloning protocol was as follows:

(a) Purification of genomic bacterial DNA from the adjuvant strain - 0065.

(b) Partial DNA digestion to create a population of 200 kb fragments for insertion into a plasmid.

(c) Production of a genomic library of the adjuvant strain (0065).

(d) The genomic library of 0065 was cloned into a plasmid.

(e) A non-adjuvant strain (with no endogenous plasmid) was transformed with the plasmid library.

(f) The amplified library will be injected into mice in the presence of 20 mg of Hb to select for clone that express the virulence trait.

(g) Viable bacteria will be recovered after 12 hours and plated. Individual colonies will be selected and tested for Hb adjuvanticity. Note: only adjuvant bacterial strains in the presence of Hb are able to survive for greater than 8 hours in the peritoneal cavity. Therefore, if we are unsuccessful in cloning the adjuvant gene (or if multiple genes are necessary for the adjuvant phenotype) there will be no viable bacteria for recovery. This project is currently being carried out by a doctoral candidate in the Experimental Pathology Program at the Albany Medical College. Support for this work will be attributed to the US Army.

DETAILED EXPERIMENTAL DESIGN

1. Cytochrome c, a protein with covalently bound heme, as an adjuvant.

Rationale: Several experimental models suggest that the state of the iron may be important in its ability to participate as an adjuvant. Previous experiments we demonstrated that haptoglobin was able to completely abrogate the hemoglobin adjuvant effect, presumably by binding and stabilizing the heme iron. Thus, we predicted that cytochrome c, a protein in which the heme is covalently bound to the protein would not serve as an adjuvant in this model.

Experimental Design: *E. coli* were grown overnight in brain heart infusion (BHI) to stationary phase, washed 3 X with sterile phosphate buffered saline (sPBS) and enumerated by measuring the optical density of a bacterial suspension at 600 nm. Male Swiss Webster (SW) mice, 18-22 g, were injected with 10^5 *E. coli* alone or with 25 mg, equivalent to ~ 2 µmol, of cytochrome c. The mortality rate was assessed after 24 hr.

Results: Cytochrome c serves as an adjuvant in the experimental infection model in a dose response that is strikingly similar to hemoglobin. On a mole to mole basis, cytochrome c appears to be an equipotent adjuvant compared with hemoglobin.

Adjuvant effect of cytochrome c: covalently bound heme

Treatment	24 hr Mortality
Control	0/10
Cytochrome c	10/10

2. Does the inhibition of macrophage nitric oxide synthase have an adjuvant-like effect ?

Rationale: An important component of macrophage killing is thought to involve the production of nitric oxide (NO•) by the enzyme nitric oxide synthase. Since NO• has been shown to interact with iron we hypothesized that the presence of hemoglobin iron might result in the reduction of the amount of NO• available for bactericidal action. Therefore, we designed an experiment to determine if the inhibition of NOS by a specific inhibitor would exert an adjuvant-like effect in animals challenged with an adjuvant strain of *E. coli*.

Experimental Design: *E. coli* 0065 was prepared as described above. Mice were injected IP with 200 µg/g body weight of the NOS inhibitor, N^6-methyl-L-arginine every 4 hours to maintain high levels (this treatment schedule was recommended by Dr. Hibbs, University of Utah, as an effective yet non-toxic dose). They were subsequently challenged with 2 x 10^4 0065 IP. The mortality after 24 hours was determined.

Results: There was no evidence that inhibition of NOS increased the virulence of the adjuvant stain of *E. coli*. This suggests but does not prove that interference with NO• production is not related to the mechanism of action of hemoglobin in the adjuvant effect.

Is the hemoglobin adjuvant effect due to the inhibition of nitric oxide synthase ?

Treatment	24 hour Mortality
Control	0/4
+ Hb	4/4
+ LAME	0/5

3. Competitive inhibition of iron-driven infection by gallium.

Rationale: Iron-driven infections may ultimately depend on the ability of the bacteria to acquire the iron. We used gallium to compete with iron uptake by bacteria.

Experimental Design: Mice were inoculated with 10^5 0065 in the presence of hemoglobin (20 mg, 1.25 µmol), ferrous sulfate (1.2 µmol) and gallium nitrate (2.7 µmol). The 24 hour mortality was assessed to determine the effect of gallium on the infection process.

Results: There was no significant effect of gallium in any combination with the various iron-containing adjuvants.

Will competitive inhibition of iron acquisition by gallium block the adjuvant effect ?

Treatment	24 hr Mortality
Control	0/5
+ Gallium	0/5
+ Hb	4/6
+ Hb + Ga	4/5
+ Fe	2/5
+ Fe + Ga	3/5

4. Do high affinity iron chelators attenuate the adjuvant effect of iron or hemoglobin ?

Rationale: When deprived of iron *E. coli* induce the synthesis of genes that enable the organism to acquire even small amounts of iron in the environment. These siderophore systems typically consist of a high affinity chelator which is relatively specific for iron and a receptor to recognize the chelate and translocate it inside the cell. Although *E. coli* do not make the siderophore deferoxamine they are able to synthesize, during periods of iron deprivation, a receptor for the deferoxamine iron complex. A family of new high molecular weight conjugates of deferoxamine has been recently described. These molecules were designed to increase the serum half life of deferoxamine. However, there high molecular weight also confers the property that these chelators would not be taken up by *E. coli*. Theoretically, iron bound to high molecular weight chelators would be unavailable for bacterial utilization. We decided to test the ability of deferoxamine and high molecular derivatives to block the adjuvant effect of iron and hemoglobin in the experimental infection model.

Experimental Design: High molecular weight deferoxamine (HMW-DFO) was generously provided by Biomedical Frontier, Inc., Minneapolis, Minnesota. Groups of mice were injected IP with either *E. coli* alone (0065) or in combination with iron or hemoglobin plus and minus DFO or HMW-DFO. Mortality was assessed at 24 hr.

Results: There was no overall protective effect of any of the iron chelators on the adjuvant effect of either iron or hemoglobin. In the case of free DFO this result is not surprising since the bacteria are able to use the chelate. However, in the case of the HMW-DFO the chelated iron should not be accessible to the bacteria. Further studies employing radiolabeled iron would have to be performed to further elucidate these results.

5. *In vitro* growth of *E. coli* in deferrated medium: the effect of iron chelates.

Rationale: Previous work on iron acquisition by bacteria has demonstrated elaborate and redundant mechanisms for acquiring iron in biological fluids which are low in available iron. This experiment was designed to determine the relative efficacy of an adjuvant strain (0065) in acquiring iron requisite for growth in a chemically defined deferrated medium.

Experimental Design: A small loop of 0065 (stationary BHI culture) was subcultured into deferrated M9 for 48 hr at which point the subculture was turbid (++++). 10^5 bacteria were placed into secondary subcultures of deferrated M9 (dM9) containing a variety of iron and chelator combinations. Turbidity was estimated after a 24 hr incubation at 37°C.

Results: The addition of iron promoted growth in all cultures regardless of the nature of the chelator present- free DFO, high molecular weight DFO or DFO cross-linked to Sepharose beads.

Growth of *E. coli* (0065) in deferrated minimal medium ± iron ± chelators.

Culture	Turbidity
Control (no *E. coli*)	-
dM9 + *E. coli*	++
dM9 + 10 μM Fe	++++
dM9 + 10 μM DFO	-
dM9 + 10 μM DFO-Fe	++++
dM9 + HMW-DFO	-
dM9 + HMW-DFO + Fe (10 μM)	++++
dM9 + Seph-DFO	++
dM9 + Seph-DFO + Fe (10 μM)	++++

6. Identification of adjuvant specific outer membrane proteins..

Rationale: Determine if there are novel outer membrane proteins produced by the adjuvant strains. The identification of such proteins would provide the beginning for unraveling the molecular basis for the iron adjuvant effect.

Experimental Design: Grow 0065 (adjuvant strain) overnight in BHI at ~ 37°C. Wash 3 X with sPBS and enumerate by optical density at 600 nm. Incubate the subcultures for 2 hr at 37°C and wash 2 X with sPBS. Compare *E. coli* extracts from BHI, serum and serum + hemoglobin cultures for one dimensional polyacrylamide gels.

Results: There are two proteins in the 20-30 kDa range found in the outer membrane protein preparations from both adjuvant strains that do not appear in outer membrane preparations from the non-adjuvant strains. These proteins may be involved with the resistance to phagocytosis that has been observed in the adjuvant strains. Further studies are underway to isolate and identify these proteins (microsequencing) to compare with known outer membrane protein sequences and in order to raise antibodies against these proteins.

7. Preincubation of 0065* in heat inactivated serum ± hemoglobin before IP injection into mice.

Rationale: If exposure to hemoglobin is sufficient to promote virulence in the adjuvant strains we may be able to determine this by preincubation in human serum in the presence and absence of hemoglobin before IP inoculation into mice.

Experimental Design: Strains 0000 and 0065 was grown overnight and counted as previously described. A subculture in human heat inactivated serum in the presence and absence of 20 mg/ml of hemoglobin for 2 hours was followed by washing the bacteria 3 X in sPBS. The mice were injected IP with 10^6 *E. coli* which is ordinarily a non-lethal dose of bacteria in the absence of hemoglobin.

Results: There was no mortality in either strain with either preincubation condition.

Preincubation of adjuvant *E. coli* strains with hemoglobin prior to peritoneal inoculation.

Treatment	24 hour Mortality
0000 preHb	0/4
0000 +Hb	4/4
0065 preHb	0/4
0065 + Hb	4/4

8. Time course of the growth of *E. coli* (adjuvant and nonadjuvant strains) *in vivo* in the presence and absence of hemoglobin.

Rationale: In an attempt to describe the natural history of the infection with adjuvant and non-adjuvant *E. coli* strains in the presence and absence of hemoglobin we sampled peritoneal lavage fluid, liver and spleen homogenates over a 12 hr time course. These experiments will help determine the dynamics of the infection and pinpoint times when major differences among the groups emerge.

Experimental Design: *E. coli* strains were grown overnight to stationary phase in BHI. The bacteria were washed 3 X in sPBS and counted. At 0 time 10^4 *E. coli* in the presence and absence of 20 mg of SF-Hb were injected IP into SW mice. The animals were sacrificed at the appropriate time either by asphyxiation with CO_2 or cervical dislocation and the 5 ml of sPBS was injected into the peritoneal cavity. The contents were mixed by 1 min of abdominal massage and the lavage fluid was carefully removed with a transfer pipette through a small abdominal incision. Additionally, the spleen and a lobe of the liver were removed, blotted, weighed and homogenized in sPBS. Appropriate dilutions of all samples were pour plated with multiple log dilution in nutrient agar and grown for 24 hr at 37°C. Colonies were counted using an Artek Automatic Plate Counter.

Results: Examination of the plates revealed that there was a striking difference in the recovery of viable bacteria between the adjuvant and non-adjuvant strains. The non-adjuvant strains were rapidly removed and/or destroyed with few bacteria remaining after only 60 min. On the other hand, the adjuvant strains demonstrated a stable number of cfu recovered from the peritoneal cavity for the first 4 - 6 hr with an increase in the cfu found in the spleen and liver. Interestingly, there was not much difference in the recovery of cfu ± hemoglobin at the 4 - 6 hr interval. However, by 10 - 12 hr there was a dramatic increase in the number of adjuvant bacteria in the presence of hemoglobin and a diminution of cfu in the absence of hemoglobin. The bacterial burden in the animals at 10 - 12 hr was above the LD_{50} for these strains.

Growth kinetics of *E. coli in vivo*

Condition	0 time	6 hours	12 hours
Peritoneal lavage			
0065 + Hb	2×10^3	~ 4×10^4	+++++
0065	4×10^3	~ 4×10^3	2
Spleen (cfu/g)			
0065 + Hb	0	4.3×10^5	2.1×10^6
0065	0	4.0×10^3	0
Liver (cfu/g)			
0065 + Hb	0	1.4×10^6	1.5×10^6
0065	0	1.0×10^3	0

Recovery of viable bacteria from the peritoneal cavity

Strain	% 0 time 2 hours	4 hours
0000*	ND	310 (+ 240)
0000*	ND	>1,000
0000* +Hb	ND	>1,000
0002*	ND	16 (+ 6)
0004	ND	0.1
0006	5	0.1
0064	7	0.2
0064pUC118	3	0.1
0065*	ND	>1,000
0065* +Hb	ND	>2,000

(ND = not determined)

9. Detection of plasmids in *E. coli* strains - 0000, 0002, 0004, 0006, 0064 and 0065 by agarose gel electrophoresis.

Rationale: Virulence factors are often encoded by plasmids. In fact, Col V is a well-known virulence plasmid found in pathogenic strains of *E. coli*. This plasmid contains the genes for the aerobactin siderophore system.

Experimental Design: All six strains of *E. coli*, 0000, 0002, 0004, 0006, 0064 and 0065 were grown as before. A standard chloramphenicol plasmid amplification protocol was employed before agarose mini-gel electrophoresis. The gels which contained a plasmid positive control were stained with ethidium bromide and observed with UV light.

Results: Staining with ethidium bromide did not show the presence of plasmids in any of the adjuvant strains. This experiment suggests that the hemoglobin adjuvant effect is not carried on a plasmid that is commonly shared by the adjuvant strains. Moreover, the lack of plasmids cleared the way for the following experiment.

10. Coinfection model with an adjuvant (0065) and a non-adjuvant strain (0064pUC118).

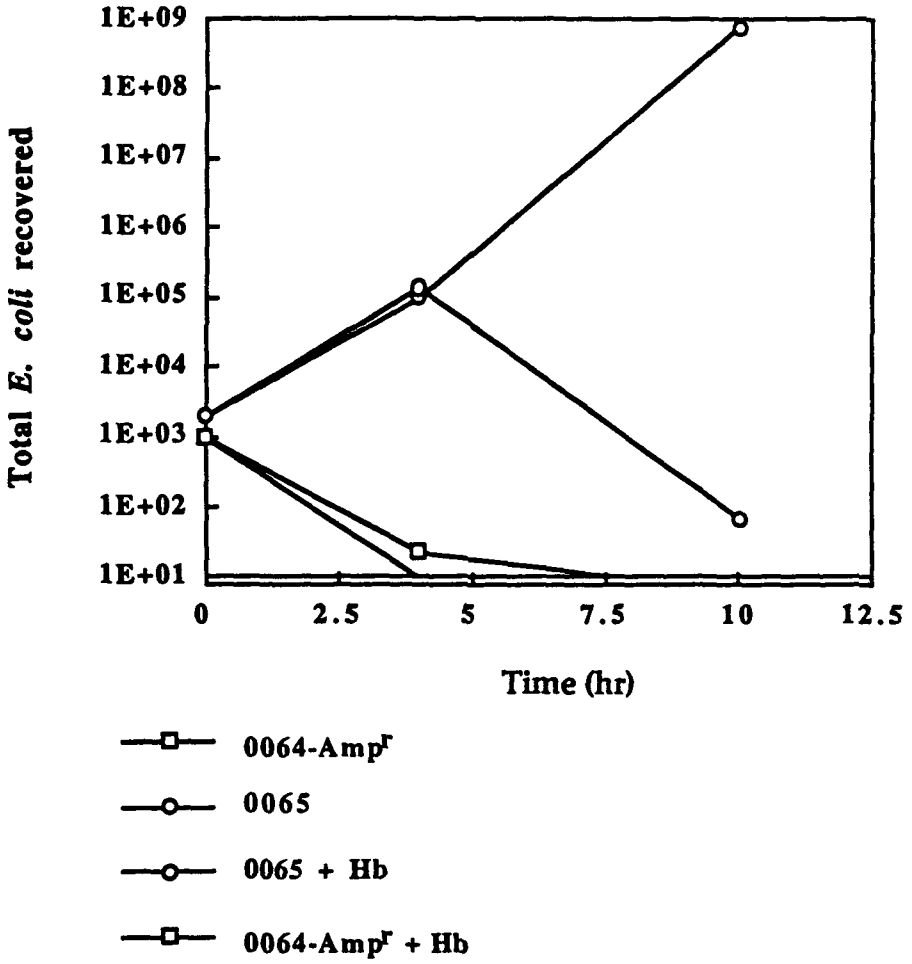
Rationale: If the adjuvant strains in the presence of hemoglobin cause a generalized impairment in host defense another bacterial strain might be able to successfully coinfect the animal. Therefore, we transformed 0064 (non-adjuvant) with pUC118 (Amp^r) by electroporation (with the kind assistance of Dr. Walter Sauerbier, University of Minnesota). By injecting equal numbers of an adjuvant and non-adjuvant (plasmid containing) at 0 time we will be able to follow the relative proportion of each bacterial strain over the time course of the infection.

Experimental Design: Transformation of 0064 with pUC118 was carried out on competent bacteria by electroporation. The plasmid-containing bacterial were selected on agar plates containing 40 μg/ml of ampicillin. 0064 (pUC118) and 0065 were grown overnight to stationary phase at 37°C washed 3 X with sPBS counted and resuspended to a concentration of 2×10^4/ml. Equal volumes of the bacterial suspensions were admixed and 1 ml was injected IP in mice in the presence and absence of SF-Hb (5 mg). The animals were sacrificed at the designated time points and the number of cfu was determined as previously described.

Results: There was a loss in the recovery of 0064 pUC118 in both the presence and absence of SF-Hb. Thus, it appears that there is no generalized impairment of host defense by the adjuvant strain in the presence of hemoglobin.

Coinfection with *E. coli* 0064pUC118 and 0065 ± SF-Hb

Strain	cfu (10 hr peritoneal lavage)
0064pUC118	0
0065	6.1×10^8 ($\pm 3.2 \times 10^8$)



11. *In vivo* bacterial growth studies ± hemoglobin.

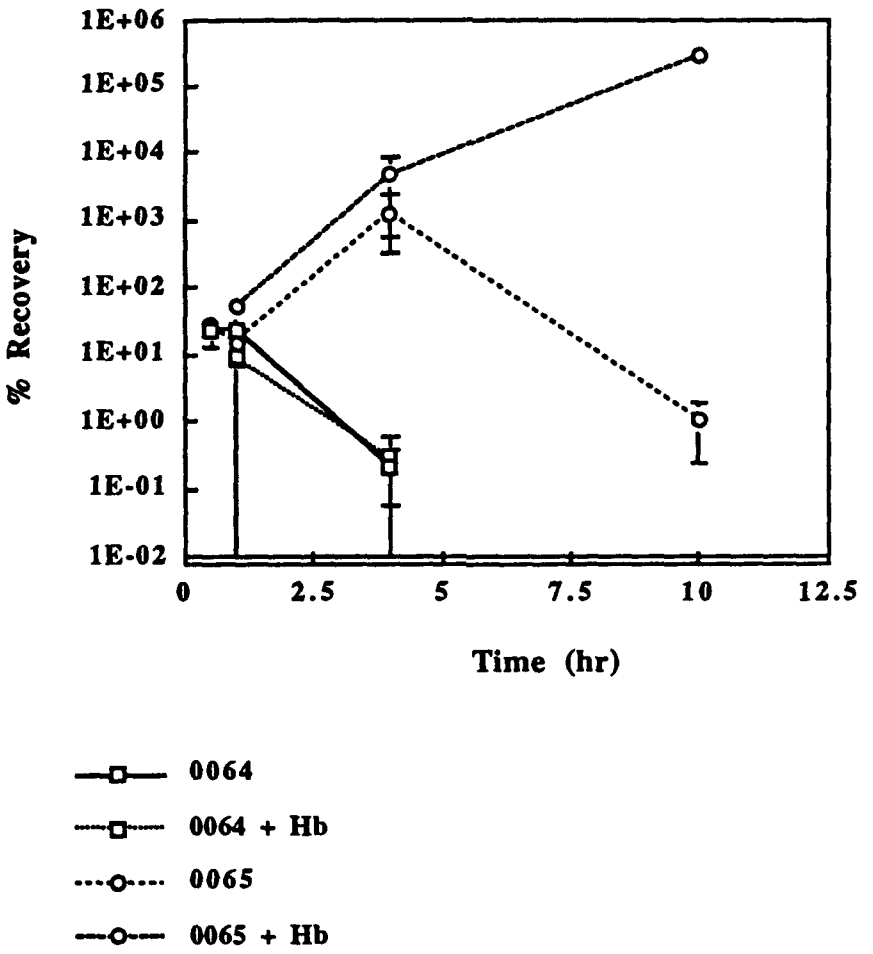
Rationale: We noted in the course of the *in vivo* time course experiments that the adjuvant strains of *E. coli* persisted in the peritoneal cavity for a number of hours at a relatively constant rate even in the absence of hemoglobin. This is in stark contrast to the rapid clearance or destruction of the non-adjuvant strains. Therefore, we decided to correlate the recovery of peritoneal cfu with the distribution of bacteria (intraphagocytic or extracellular) in the presence and absence of Hb.

Experimental Design: Bacterial strains were grown and enumerated as before. In order to have enough bacteria to detect on slides following cytospin 10^6 bacteria were injected per animal. At either 30 min or 4 hr the animals were sacrificed, as before, and 2.5 ml of cold sPBS was injected IP. Following mixing of the lavage by abdominal massage the fluid was removed and plates and slides were prepared.

Results: In general, there was a strong correlation between the loss of cfu and the non-adjuvant strains. By 30 min there was a marked reduction in the number of recoverable bacteria. Examination of the slides revealed that vast majority of the non-adjuvant bacteria were intracellular, mostly in macrophages. Fewer of the adjuvant bacteria were intracellular, but there was still ~ 50% phagocytosis by peritoneal macrophages at 30 min.

There was a good correlation between the plate counts and the slides from the adjuvant strains after several hours. At 4 hr there were numerous viable bacteria in the peritoneal lavage (~3 - 10 fold increase form the 0 time inoculum). This corresponded a large number of bacteria on the slides. Most importantly, there were very few adjuvant bacteria that were internalized by the macrophages at 4 hr. Some bacteria were observed in PMN but most were extracellular. Surprisingly, the presence of SF-Hb did not have a major effect on whether or not the non-adjuvant or adjuvant bacteria were phagocytosed at either time interval.

Another correlation was observed between the magnitude of the SF-Hb effect on the LD_{50} of the adjuvant strains and the ability to persist in the peritoneal cavity. Namely, strain 0002 is the weakest of the adjuvant strains and shows the least amount of growth in the peritoneal cavity at the 4 hr time point.



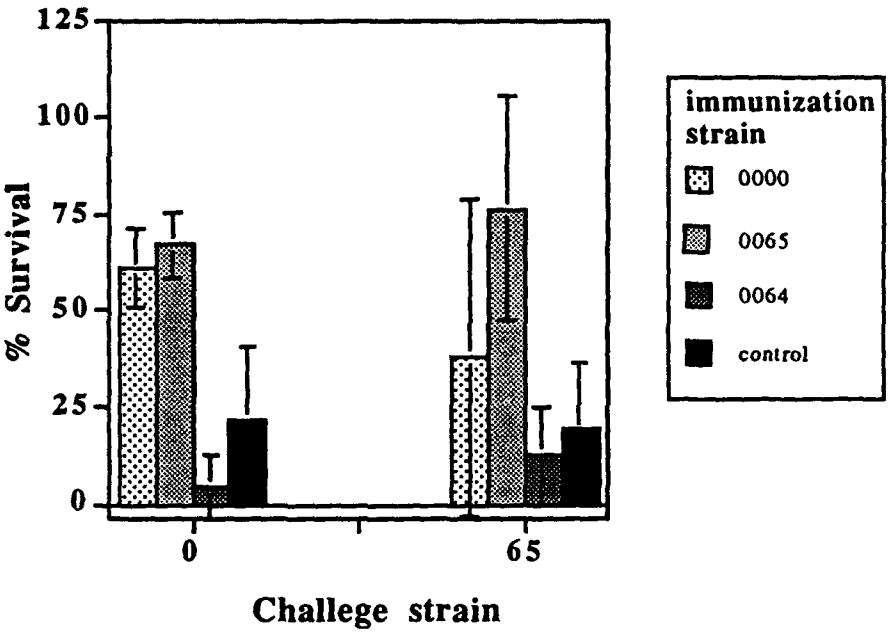
12. Autologous and cross immunization with non-adjuvant and adjuvant strains.

Rationale: If the adjuvant bacteria contain a novel surface determinant that interferes with phagocytosis, an antibody to this determinant may neutralize the adjuvant phenotype. Antiserum raised against non-adjuvant bacteria should not affect the infection. In addition, if the determinant was the same in all adjuvant strains antiserum raised against one adjuvant strains should cross-react with another stain and neutralize the adjuvant phenotype.

Experimental Design: Mice were immunized against adjuvant (0000, 0065) or non-adjuvant strains (0064) of heat killed intact *E. coli*. Animals were injected with ~ 10^7 dead organisms subcutaneously. This procedure has been shown to maximize the production of antibodies directed against the outer membrane determinants.

Two weeks after the initial immunization the animals were boosted and the experimental infections were performed 5-7 days thereafter.

Results: Survival of an injection of 10^6 *E. coli* in the presence of 20 mg of Hb was carried out using both adjuvant strains of *E. coli*. When strain 0000 was injected into naive (control) mice there was the expected 25% mortality after 24 hr. Also, when the same number of 0000 were injected into mice immunized against 0064 (non-adjuvant) there was a similar high mortality. However, when mice immunized against 0000 or 0065 were used there was a statistically significant improvement in the survival. The fact that mice immunized against 0065 were protected against lethal infection by 0000 suggests that they share similar antigenic determinants that are important in mediating the infection.



13. Molecular cloning and selection of the virulence gene in hemoglobin-adjuvant strains of *E. coli*.

Rationale: Molecular cloning can be an effective technique to isolate and identify specific virulence genes. We will generate a genomic DNA library from one of the adjuvant strains of *E. coli* (0065). Approximately 200 kb fragments will be cloned into a plasmid vector. A non-adjuvant strain (004) will be transformed with these plasmids. The transformants will be amplified and injected IP in the presence of Hb in our mouse model of experimental peritonitis. Assuming that we are successful with the molecular cloning we should be able to recover any bacteria expressing the adjuvant gene after 12 hr. We know from our previous work that

non-adjuvant bacteria are rapidly cleared from the peritoneal cavity which is sterile by 12 hr. Therefore, we will use the host defense system to select for any transformants that have acquired the adjuvant trait.

Experimental Design: Genomic DNA was isolated from the adjuvant strain and subjected to partial restriction enzyme digestion (BamH1). Fragments of approximately 200 kb were isolated using agarose electrophoresis. The 200 kb fragments were inserted into pUC19 plasmids. A non-adjuvant strain of *E. coli* was transformed and amplified. The selection of transformants expressing the adjuvant phenotype will be selected *in vivo*. Approximately 10^6 *E. coli* transformants (0004) are injected in the presence of 20 mg of Hb. After 12 hr the mice will be examined for signs of infection. Animals will be injected IP with 2.5 ml of sterile PBS, sacrificed and the peritoneal lavage will be plated on nutrient agar. Colonies will be isolated and grown overnight in BHI. After washing, approximately 10^6 *E. coli* + 20 mg of Hb will be injected IP in mice. A peritoneal lavage after 12 hr should recover only bacteria that are derived from transformants containing the adjuvant gene.

Results: We made a genomic library from an adjuvant strain of *E. coli* (0065) and have transformed and amplified a non-adjuvant strain of *E. coli* (0004). We are currently attempting to select for a transformant that exhibits the adjuvant phenotype. If we are successful, we will recover the plasmid from the transformant. At that point we will apply basic techniques in genetics and molecular biology to identify the DNA sequence responsible for conferring the adjuvant phenotype.

CONCLUSION

These experiments clearly demonstrate that chemically cross-linked Hb (DBBF-Hb) is as effective on a mole to mole basis at promoting lethal *E. coli* infection in an animal model of experimental bacterial peritonitis. Whereas, only a fraction of human clinical isolates (3 out of 23) demonstrated markedly enhanced virulence in the presence of hemoglobin-iron, the extreme pathogenicity of these infections suggests that the purposeful infusion of free hemoglobin into an injured patient could have disastrous consequences. The precise molecular mechanism of pathogenesis of these hemoglobin-iron-driven infections has not yet been elucidated.

The most promising results were obtained from relatively simple experiments in which we determined the rate of growth of both adjuvant and non-adjuvant strains *in vivo* in the presence and absence of hemoglobin. These data suggest that the adjuvant strains are inherently more resistant to phagocytosis *in vivo* than the non-adjuvant strains; this is in spite of similar results in *vitro* PMN killing of 0064 and 0065. The concept of impairment of host defense was not supported by the results of our coinfection study in which we demonstrated that the non-adjuvant strain, containing an ampicillin resistance plasmid, was cleared and/or destroyed at the same rate in the presence of the adjuvant strain and hemoglobin as when it was injected alone.

Thus, it appears that the hemoglobin adjuvant effect is consistent with the following mechanism. First, the adjuvant strains of bacteria appear to be more resistant to phagocytosis than non-adjuvant strains. Second, since they are able to resist phagocytosis hemoglobin-iron determines the outcome of the infection. In

other words, the nonadjuvant bacteria are not able to utilize the hemoglobin -iron because they are cleared from the peritoneal cavity too quickly. Thus, the nutritional effect of iron is most pronounced several hours after the beginning of the infection.

We are confident that using the multiple approach of cloning the adjuvant gene, identifying a novel outer membrane protein(s) and raising antibodies that block the adjuvant phenotype that we will be able to elucidate the molecular mechanism for this dramatically enhanced virulence in the presence of hemoglobin-iron. This would enable us to determine how wide spread this trait is amongst pathogenic *E. coli* . We would also have the tool to determine if other gram negative bacteria also use this or a closely related virulence mechanism.

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